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Caffeine compromises proliferation of human hippocampal progenitor cells

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30

Abstract

The age-associated reduction of adult hippocampal neurogenesis (AHN), the formation and integration of new neurons in the adult hippocampus, has been associated with cognitive decline. Numerous factors have been shown to modulate this process, including dietary components. Frequent consumption of caffeine has been correlated with an increased risk of cognitive decline, but further evidence of a negative effect on AHN are limited to animal models. Here, we used a human hippocampal progenitor cell line to investigate the effects of caffeine on hippocampal progenitor integrity and proliferation. The effects of five caffeine concentrations (0mM=control, 0.1mM~150mg, 0.25mM~400mg, 0.5mM~750mg, and 1.0mM~1500mg) were measured following acute (1 day) and repeated (3 days) exposure. Immunocytochemistry was used to quantify hippocampal progenitor integrity (i.e., SOX2- and Nestin-positive cells), proliferation (i.e., Ki67-positive cells), cell count (i.e., DAPI-positive cells), and apoptosis (i.e., CC3-positive cells). We found that progenitor integrity was significantly reduced in supraphysiological caffeine conditions (i.e., 1.0mM~1500mg), but relative to the lowest caffeine condition (i.e., 0.1mM~150mg) only. Moreover, repeated exposure to supraphysiological caffeine concentrations (i.e., 1.0mM~1500mg) was found to affect proliferation, significantly reducing %Ki67-positive cells relative to control and lower caffeine dose conditions (i.e., 0.1mM~150mg and 0.25mM~400mg). Caffeine treatment did not influence apoptosis and there were no significant differences in any measure between lower doses of caffeine (i.e., 0.1mM, 0.25mM, 0.5mM) - representative of daily human caffeine intake - and control conditions. Our study demonstrates that dietary components such as caffeine can influence AHN and may be indicative of a mechanism by which diet affects cognitive outcomes.

1 Introduction

Adult hippocampal neurogenesis (AHN), the formation of new neurons from neural progenitor cells, has recently regained considerable attention, particularly in the human hippocampus (Kempermann et al., 2018; Lucassen et al., 2020). This highly vascularized ‘neurogenic niche’, retains developmental signals and morphogens that influence cell proliferation, differentiation, and survival throughout life (Gonçalves et al., 2016; Spalding et al., 2013). The rates at which these processes occur have been associated with hippocampal-dependent learning and memory functions (Cleland et al., 2009; Sahay et al., 2011; Snyder et al., 2005) and this association is particularly interesting when considering ageing and cognitive decline, during which hippocampal function typically deteriorates (Small et al., 2002). Moreover, neural progenitor proliferation declines in rodents as ageing progresses (Heine et al., 2004; Rao et al., 2006) and this has been strongly correlated with impaired performance in spatial memory and learning tasks (Sahay et al., 2011; Villeda et al., 2011).

This association with cognitive decline presents AHN as a unique target area for preventative interventions. Accordingly, rescuing later life neurogenesis has recently gained interest and a focus has been given to the factors that modulate neurogenesis (Baptista and Andrade, 2018). While neurogenesis is facilitated by the neurogenic niche, it is not only central nervous system-derived signals that influence AHN. Indeed, AHN is also modulated by both the external environment (Lledo et al., 2006) and the system milieu (Villeda et al., 2011; Yousef et al., 2019). For example, stress and sleep deprivation have been shown to reduce AHN (Gould et al., 1998; Hairston et al., 2005; Lucassen et al., 2010), while running increases neurogenesis (van Praag et al., 2005). Moreover, these environmental factors have been similarly correlated with spatial learning and memory (Nilsson et al., 1999; Oomen et al., 2010; 2014), highlighting the possibility of leveraging behavioral interventions to target the neurogenic process and, consequently, cognitive ability.

Diet is another environmental factor that has been shown to influence the neurogenic process (Abbink et al., 2020; Miquel et al., 2018; Stangl and Thuret, 2009). *Drosophila* research shows that nutritional factors can influence the exit of neural hippocampal progenitors from quiescence (Chell and Brand, 2010; Spéder and Brand, 2014), and other nutritional-based changes to the hippocampal progenitor pool have been likewise demonstrated across other species (Cavallucci et al., 2016; Sakayori et al., 2013; Spéder et al., 2011). For instance, in humans, the nutrient-sensing pathways: the mammalian target of rapamycin (mTOR), sirtuin, and insulin-like growth factor 1, have all been associated with hippocampal progenitor maintenance (de Lucia et al. 2020). However, the influence of nutrition and meal content on the hippocampal progenitor pool occurs in a complex manner, with the nature of change dependent on the food groups consumed. For instance, a high fat diet has been shown to decrease proliferation in rats (Lindqvist et al., 2006), while in contrast omega-3 fatty acids increase proliferation in lobsters (Beltz et al., 2007). Interestingly, these changes to proliferation directionally correspond with their associated cognitive outcomes, as omega-3 has been shown to improve cognitive outcomes, while high fat diets impair cognitive performance (Fotuhi et al., 2009; Witte et al., 2009; Winocur and Greenwood, 2005; Yam et al., 2019). Thus, the variable nature of meal content and its influence on AHN may provide a flexible and unique mechanism of regulating the neurogenic process within the human population. However, further defining the dietary components that affect the neurogenic process and their direction of influence is crucial before such dietary-based interventions can be developed.

Caffeine, the most widely consumed psychostimulant in the world (Ferré, 2016), has been widely implicated as a cognitive modulator (Glade, 2010; Rosso et al., 2008). Caffeine consumption has traditionally been argued to produce health benefits on a neurological basis, including protection against cognitive decline in women aged over 65 years (Arab et al., 2011; Ritchie et al., 2007). However, we recently demonstrated a negative effect of caffeine on cognition, identifying caffeine as one of 22 metabolites predictive of cognitive decline in an ageing population, over a 13-year period (Low et al., 2019). Further evidence to support a negative effect of caffeine comes from animal models that focus on AHN. Specifically, when administered chronically, physiologically relevant doses of caffeine decreased neuronal precursor proliferation in rats (Wentz and Magavi, 2009), which was further correlated with impaired hippocampal-dependent learning and memory (Han et al., 2007). However, due to *in vivo* imaging constraints (Ho et al., 2013), the effect of caffeine on the human neurogenic process has not yet been explored. With the mixed clinical evidence on the impact of caffeine on cognitive decline and its large-scale consumption worldwide, further investigation is warranted. Determining the effects of caffeine on the neurogenic process, and ultimately cognition, will contribute to our understanding of how diet affects these phenomena, which could assist in the development of appropriate interventions.

Therefore, this study investigated the effects of caffeine on the human neurogenic process, focusing specifically on proliferation and hippocampal progenitor integrity. We used a human hippocampal progenitor cell line, for the first time, to investigate, (i) the effects of five caffeine concentrations, and (ii) the effects of acute and repeated exposure to caffeine – all on hippocampal progenitor integrity and proliferation.

2 Materials and Methods

2.1 Cell Line and Culture Conditions

The human hippocampal progenitor cell line *HPC0A07/03* (HPC; ReNeuron Ltd, Surrey, UK) was used in all experiments, as previously described (de Lucia et al., 2020; Smeeth et al., 2020). Briefly,

cells were cultured in reduced modified medium (RMM), namely Dulbecco's Modified Eagle's Media/F12 (DMEM:F12, Sigma), supplemented with 0.03% human albumin solution (Zenalb), 100 µg/mL human apo-transferrin, 16.2 µg/mL human putrescine diHCl, 5 µg/mL human recombinant insulin, 60 ng/mL progesterone, 2 mM L-glutamine and 40 ng/mL sodium selenite. For proliferation, the medium also included 10 ng/mL human basic fibroblast growth factor (bFGF), 20 ng/mL human epidermal growth factor (EGF) and 100 nM 4-hydroxytamoxifen (4-OHT). Cells were grown on tissue culture flasks (Nunc, Denmark), incubated at 37°C, 5% CO₂ and saturated humidity, and were routinely passaged at 80% confluency before being plated for experiments.

2.2 Proliferation Assay

The HPC proliferation assay was carried out as previously described (de Lucia et al., 2020; Smeeth et al., 2020). Briefly, HPCs were seeded into two 96-well plates (Nunc, Denmark) per experiment: one plate for acute (one-time) caffeine treatment, the other for repeated caffeine treatment. Plates were seeded at a density of 1.2×10^4 , at P21 in caffeine-free proliferation media, with three technical replicates and three biological replicates. All cells, excluding the control conditions, received caffeine treatment 24 hours after seeding. Cells undergoing acute treatment were left undisturbed for 48 hours, while cells undergoing repeated exposure received another caffeine treatment 24 hours after the initial treatment. Control conditions were incubated in caffeine-free proliferation media in all instances. Seventy-two hours after seeding, all plates were washed and fixed as previously described (de Lucia et al., 2020; Smeeth et al., 2020). **Figure 1** depicts the assay timeline as per the two exposure conditions. For details on the proliferation assays and fixation methods see **Supplementary material**.

2.3 Caffeine Treatments

Caffeine (5g) was obtained from Sigma (MO, USA) in powdered form, with a molecular weight of 194.19 g/mol. Caffeine conditions were as follows: control (no caffeine, media only); low (0.1mM, ~150mg, ~1 cup); moderate (0.25mM, ~400mg, ~2-3 cups); high (0.5mM, ~750mg, ~5 cups); and supraphysiological (1.0mM, ~1500mg, ~10 cups), reflecting human intake habits and previous animal models (EFSA, 2015; Wentz and Magavi, 2009). Caffeine concentrations were calculated based on previous research stating that 150mg of caffeine, the mean caffeine content of a Starbucks cappuccino (Ludwig et al., 2014), is approximately equivalent to 0.1mM (Su et al. 2013a; 2013b). For full details on the caffeine treatments see **Supplementary material**.

2.4 Immunocytochemistry

Cell count, progenitor cell integrity, progenitor proliferation and cell death were visualized using 4',6-diamidino-2-phenylindole (DAPI), Nestin and SRY-Box Transcription Factor 2 (SOX2), Ki67, and cleaved caspase-3 (CC3), respectively, using immunocytochemistry as previously described (de Lucia et al., 2020; Smeeth et al. 2020). For protocol details, antibodies used, and representative images see **Supplementary material**.

2.5 Image Analysis

Immunostainings were quantified using CellInsight NXT High Content Screening (HCS) platform (ThermoScientific) and the HCS Studio Cell Analysis Software (Thermo Scientific), as previously described (de Lucia et al., 2020; Smeeth et al. 2020). This software quantifies the intensity of fluorescent stainings of each marker in user-defined regions, against the identification of individual

cells by nuclear staining. For details on the protocols and parameters used see **Supplementary material**.

2.6 Statistical Analyses

Data analyses were conducted using IBM SPSS Statistics 26 (IBM Ltd., Portsmouth, UK). All data were assessed for normality using probability-probability plots and the Kolmogorov-Smirnov test, and for homogeneity of variance using the Levene's test. For data that did not conform to normality and/or homoscedasticity non-parametric statistical tests were applied. To evaluate differences between DAPI, Ki67, C33, and Ki67/CC3, a two-way analysis of variance (ANOVA) with a Bonferroni post hoc correction was applied. To evaluate differences in SOX2, Nestin and Nestin/SOX2 a series of Kruskal Wallis tests with Dunn's post hoc corrections were applied. All tests carried out were two-sided and the alpha criterion used was $p < .05$. Data are represented as the mean (M) and standard error of the mean (SEM), or the median (Mdn) and interquartile range (IQR).

3 Results

3.1 Exposure to Caffeine Reduces Cell Number

There was no significant interaction of caffeine concentration and exposure type, i.e., repeated versus acute caffeine treatment, on cell number, as measured by DAPI-positive cell density ($p = .947$), nor was there a significant main effect of exposure ($p = .580$). However, as shown in **Figure 2.A**, there was a main effect of caffeine concentration on DAPI-positive cell density ($p = .036$), such that higher caffeine doses reduced cell number. However, due to issues of power, post hoc analyses revealed no specific differences between any of the caffeine conditions, but a trend for significance did emerge with respect to the supraphysiological dose (i.e., 1.0mM~1500mg) relative to the lowest caffeine dose (i.e., 0.1mM~150mg) with a 58.6% reduction in cell count observed ($p = .058$).

3.2 Exposure to Supraphysiological Caffeine Concentrations Reduces Hippocampal Progenitor Integrity Compared with Lower Caffeine Doses Only

There was no significant main effect of exposure on hippocampal progenitor integrity, as measured by both %Nestin-positive ($p = .901$) and %SOX2-positive ($p = .917$) cells. However, as shown in **Figure 3**, there was a significant main effect of caffeine concentration on both %Nestin-positive ($p = .034$) and %SOX2-positive cells ($p = .016$), all while controlling for cell number.

Post hoc analyses revealed that the supraphysiological caffeine concentration (i.e., 1.0mM ~ 1500mg) significantly reduced the %Nestin-positive cells by 1.5% relative to the lowest caffeine concentration (i.e., 0.1mM ~ 150mg; $p = .016$; **Figure 3.A**). No significant differences in %Nestin-positive cells for any of the caffeine concentrations relative to control were observed (0.1mM~150mg: $p > .99$; 0.25mM~400mg: $p > .99$; 0.5mM~750mg: $p > .99$; 1.0mM~1500mg: $p = .388$). However, it should be noted that the supraphysiological caffeine dose was reduced relative to control conditions but did not survive multiple comparison correction (non-adjusted $p = .039$).

Similar to Nestin data, post hoc analyses of %SOX2-positive cells revealed that the supraphysiological caffeine dose (i.e., 1.0mM ~ 1500mg) significantly reduced %SOX2-positive cells by 2.3%, again, relative to the lowest caffeine concentration (0.1mM~150mg, $p = .013$; **Figure 3.C**). Moreover, there was a trend for a significant reduction in %SOX2-positive cells in the supraphysiological caffeine dose relative to the moderate caffeine concentration, i.e., 0.25mM~400mg, ($p = .059$). Again, no significant differences were observed relative to control

conditions (0.1mM~150mg: $p = >.99$; 0.25mM~400mg: $p > .99$; 0.5mM~750mg: $p > .99$; 1.0mM~1500mg: $p = .304$), however like with the Nestin data, %SOX2-positive cells in the supraphysiological caffeine condition were reduced relative to control but did not survive multiple comparison correction (non-adjusted $p = .03$).

Unsurprisingly, a similar results pattern was observed for %Nestin/SOX2-positive cells. Specifically, no significant main effect of exposure ($p = .868$) was observed, but there was a significant main effect of caffeine on %Nestin/SOX2-positive cells ($p = .021$), with the supraphysiological concentration reducing %Nestin/SOX2-positive cells by 2.1% relative to the lowest caffeine concentration only ($p = .016$; **Figure 3.E.**). Moreover, the %Nestin/SOX2-positive cells for the supraphysiological dose was reduced compared with the control condition (non-adjusted $p = .029$) and the moderate caffeine concentration, i.e. 0.25mM~400mg (non-adjusted $p = .008$) but these did not survive multiple comparison correction.

3.3 Repeated Exposure to Supraphysiological Caffeine Concentrations Reduces Hippocampal Progenitor Proliferation

There was no significant interaction effect of caffeine and exposure on proliferation, as measured by the percentage of Ki67-positive cells ($p = .102$). However, as shown in **Figure 4.A**, there was both a significant main effect of exposure ($p = .009$) and caffeine concentration ($p < .001$) on the %Ki67-positive cells, all while controlling for cell number. Specifically, repeated exposure to the supraphysiological caffeine concentration (i.e., 1.0mM ~ 1500mg) significantly reduced proliferation by 37% relative to control conditions ($p = .001$), by 39.5% relative to the lowest caffeine dose (i.e., 0.1mM ~150mg; $p < .001$), and by 37.7% relative to the moderate caffeine dose (i.e., 0.25mM ~ 400mg; $p = .001$). No significant differences were found between the control condition and the other caffeine concentrations, i.e., 0.1mM ($p > .99$), 0.25mM ($p > .99$), and 0.5mM ($p = .446$), nor were any significant differences observed for acute exposure, that is, a single, one-time caffeine treatment.

3.4 Exposure to Caffeine does not Affect Apoptosis

As depicted in **Figure S2.A**, there was no significant interaction of caffeine concentration and exposure on apoptosis, as measured by %CC3-positive cells ($p = .616$), nor was there a significant main effect of exposure ($p = .571$) or caffeine concentration ($p = .474$) – all while controlling for cell number. Furthermore, as shown in **Figure S2.C**, there was no significant interaction of caffeine concentration and exposure on proliferative cell death, as measured by %Ki67/CC3-positive cells, ($p = .797$), nor was there a significant main effect of exposure ($p = .759$) or caffeine concentration ($p = .167$) – again, all while controlling for cell number.

4 Discussion

In this study we explore the effects of acute and repeated caffeine exposure at different concentrations on hippocampal progenitor proliferation and integrity, using an *in vitro* hippocampal cellular model. We demonstrate that a repeated supraphysiological dose of caffeine, i.e., 1.0mM~1500mg or ~10 cups of coffee, significantly reduces progenitor proliferation, as measured by %Ki67-positive cells, relative to the control condition (no caffeine) and to both the lowest (i.e., 0.1mM~150mg or ~1 cup) and moderate (i.e., 0.25mM~400mg or 2-3 cups) caffeine concentrations. Moreover, the supraphysiological dose (~10 cups of coffee), whether acutely or repeatedly administered, negatively influences progenitor integrity, as measured by both %Nestin- and %SOX2-positive cells, but only when compared with the lowest caffeine dose (~1 cup of coffee). Finally, we show that caffeine, irrespective of the degree of exposure or concentration, does not affect overall, or

proliferative, cell death, as measured by %CC3-positive cells and %Ki67/CC3-positive cells, respectively.

Our finding that repeated treatment with a supraphysiological caffeine concentration, that is, intake of ~10 cups of coffee, reduces hippocampal progenitor proliferation directly contrasts previous findings from Wentz and Magavi (2009), who used an animal model and observe that supraphysiological doses increase proliferation. However, this inconsistency could be attributed to differences in study design; all previous findings were from an animal model, and therefore are not entirely translatable to our own study design that uses a human *in vitro* cellular model. Furthermore, our study found no effect of lower caffeine doses on hippocampal neurogenesis, despite previous literature demonstrating a decrease in proliferation (Han et al., 2007; Wentz and Magavi, 2009). While the discrepancies between our findings and that of the previous literature could be a consequence of the different models used, it is more likely attributable to the different timescales investigated. While our study investigated repeated exposure over 72-hours of proliferation, Wentz and Magavi (2009) and Han et al. (2007) investigated caffeine exposure over seven days and four weeks, respectively. In the context of our work, while the supraphysiological caffeine concentration is strong enough to produce a detrimental effect over a short period of time, our 72-hour paradigm may be insufficient to replicate the results seen from chronic exposure with lower, more physiologically relevant doses. Therefore, future work should seek to extend our paradigm to explore the longer-term effects of chronic, rather than repeated treatment, with physiologically relevant caffeine concentrations.

Previously unexplored within an *in vitro* model of HPCs, our findings relating to %Nestin- and %SOX2-positive cells may provide some insight into the mechanisms by which the supraphysiological caffeine dose influences proliferation. SOX2 has been implicated as an important requirement for the maintenance of self-renewal and pluripotency in human embryonic hippocampal progenitors (Fong et al., 2008), and this has been further demonstrated in adult neural hippocampal progenitors. Ferri et al. (2004) found that knocking down SOX2 leads to reduced proliferation and a depletion of the neural hippocampal progenitor pool – a finding seemingly consistent with our own. Indeed, we report a reduction in both %Nestin-, %SOX- and %Nestin/SOX2-positive cells, and simultaneously find no change in either the total %CC3-positive cells or %CC3/Ki67-positive cells (i.e., specifically proliferative cell death), suggesting that the observed decrease in proliferation following repeated supraphysiological caffeine treatment likely stems from changes in the hippocampal progenitor pool itself. Moreover, these early changes to the hippocampal progenitor pool may produce prolonged knock-on effects on differentiation, that not only include reduced neurogenesis but also morphological abnormalities of the resulting neurons (Cavallaro et al., 2008). While we find no statistically significant effect of supraphysiological caffeine doses on SOX2 relative to control conditions, we believe that this could potentially be due to issues of power (Cremers et al., 2017), given that prior to post hoc adjustment, the supraphysiological concentration of ~10 cups of coffee shows a reduction in both %Nestin-, %SOX2- and %Nestin/Ki67-positive cells, all relative to control conditions. Furthermore, it is notable that hippocampal progenitor integrity was statistically assessed using non-parametric methods, which are typically less powerful than parametric equivalents (Siegel, 1957). Therefore, it would be highly profitable for future research to include a greater sample size to more fully elucidate the effect of supraphysiological caffeine concentrations on hippocampal progenitor integrity.

The precise mechanisms by which caffeine affects neurogenesis are widely unknown, but the observed changes to %SOX2-positive cells may provide some insight. Caffeine has commonly been associated with protein kinase B (PKB or Akt) signaling; specifically, it has been attributed to downregulating Akt signaling in a wide range of cell types, from HeLa to mouse epidermal cell lines

(Nomura et al., 2005; Saiki et al., 2011). Pertinently, Akt signaling has been linked with SOX2, having been shown to promote the expression of SOX2 adult hippocampal neural progenitor cells (Peltier et al., 2010). Furthermore, Akt signaling itself decreases with age, akin to SOX2 expression and AHN, but its reactivation has been shown to ameliorate age-related defects in neuronal development (Tang et al., 2019). It is therefore possible that our finding of reduced %SOX2-positive cells following supraphysiological caffeine treatment is a product of downregulated Akt signaling. To our knowledge, the effect of caffeine on Akt signaling within an HPC cell line has not yet been investigated, and therefore future research would be instrumental in validating a link between caffeine and SOX2 expression in HPCs and revealing whether this action could be mediated by Akt signaling.

While our work reveals a negative effect of supraphysiological caffeine on human hippocampal progenitor integrity and proliferation, there are some limitations in that our model that may have influenced the extent to which caffeine affects the neurogenic process. For example, caffeine is metabolized in the liver by the enzyme CYP1A2, which accounts for approximately 90% of caffeine metabolism (Arnaud, 2011). Interestingly, a C/A polymorphism in intron 1 of the CYP1A2 gene appears to affect CYP1A2 enzymatic activity, and ultimately alter the rate of caffeine metabolism (Sachse et al., 2001). Indeed, Butler et al., (1992) defined CYP1A2 activity as being trimodally distributed, with slow, intermediate, and rapid metabolizers, as determined by caffeine urinary metabolite analyses. Essentially, the rates of caffeine clearance differ depend on an individual's genetic variant, and therefore the amount of time that caffeine is present in the systemic environment is subject to interindividual differences. These differences in caffeine metabolic rates have been associated with differences in the risk of some neurodegenerative diseases, with individuals possessing the C allele, i.e., slow metabolizers, having decreased caffeine-related risk of Parkinson's Disease (Chuang et al., 2016; Popat et al., 2011). Therefore, it is possible that this polymorphism may also mediate differences in the way caffeine affects AHN, especially considering that caffeine reaches the brain via the systemic environment. Our study measures the direct effect of caffeine exposure on hippocampal progenitor cells, without accounting for differential metabolic rates in the liver caused by the CYP1A2 polymorphism.

Furthermore, while the caffeine concentrations used in our study reflect 'intake', this is not representative of peak plasma levels obtained following caffeine metabolism. Indeed, around 99% of caffeine is metabolized into paraxanthine, theobromine, and theophylline (Arnaud, 1993; Nehlig, 2018) and, thus, only residual caffeine remains in the systemic environment. For instance, consumption of 160mg of caffeine, in the form of a hot coffee, was shown to produce an average peak plasma level of 3.74 μ g/mL, or 19.26 μ M, in humans (White Jr et al., 2016). The lowest caffeine concentration in our study, 0.1mM, represents approximately 150mg of caffeine (Su et al., 2013a; 2013b), or one Starbucks cappuccino (Ludwig et al., 2014), whereas plasma caffeine levels typically reach between 20 to 50 μ M (Graham, 2001). Therefore, the levels of caffeine tested in this study reflect supranutritional doses, not the physiologically relevant concentrations that would reach the neurogenic niche *in vivo*. However, this study provides proof of concept that caffeine can modulate the neurogenic process, and it would be profitable for future research to investigate the effects of nutritional and supranutritional caffeine concentrations on neurogenesis over time.

Finally, although our aim was to explore the effect of caffeine on hippocampal progenitor cells, given that diet has been shown to specifically influence neural hippocampal progenitor behavior (Sakayori et al., 2013; Spéder et al., 2011), our work only narrowly focuses on the neurogenic process. By only investigating proliferation, we do not know what longer-term, knock-on effects might arise from caffeine treatment, with respect to differentiation and/or survival. Therefore, future work should seek

to extend our paradigm to also evaluate the impact of caffeine on differentiation and neuron morphology.

However, despite these limitations to our work, this study is the first, to our knowledge, to investigate the direct effects of caffeine on the neurogenic process using a human *in vitro* cellular model. Neurogenesis is influenced by a range of systemic and environmental factors that are difficult to control for in an *in vivo* environment (Azari and Reynolds, 2016) – an issue that is mostly controlled for in *in vitro* models. Moreover, species and strain differences have long been a criticism of animal models (Martić-Kehl et al., 2012), and AHN in particular has been shown to widely differ amongst mammalian species (Amrein et al., 2011). Therefore, our work investigates the direct effect of caffeine the human neurogenic process and is thus likely to yield results with greater translational value.

In summary, our study demonstrates that dietary components such as caffeine can influence hippocampal neurogenesis and may be indicative of one mechanism by which diet affects cognitive outcomes. However, future research that (i) further explores the effects of human consumption-related caffeine doses on neural progenitor proliferation and differentiation, and (ii) correlates this with cognitive outcomes, are needed to validate an association with caffeine, AHN, and cognitive decline.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

Vikki Houghton: Designed and conceptualized study; data collection; statistical analysis; data interpretation; drafted and revised the manuscript for intellectual content.

Andrea Du Preez: Designed and conceptualized study; data collection supervision; statistical analysis; data interpretation; drafted and revised the manuscript for intellectual content.

Sophie Lefèvre-Arbogast: Statistical analysis; data interpretation; revised the manuscript for intellectual content.

Chiara de Lucia: Data interpretation; revised the manuscript for intellectual content.

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374 Claudine Manach: Data interpretation; revised the manuscript for intellectual content.

375 Sandrine Thuret: Designed and conceptualized study; statistical analysis; interpreted the data; drafted
376 and revised the manuscript for intellectual contact.

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386 **9 References**

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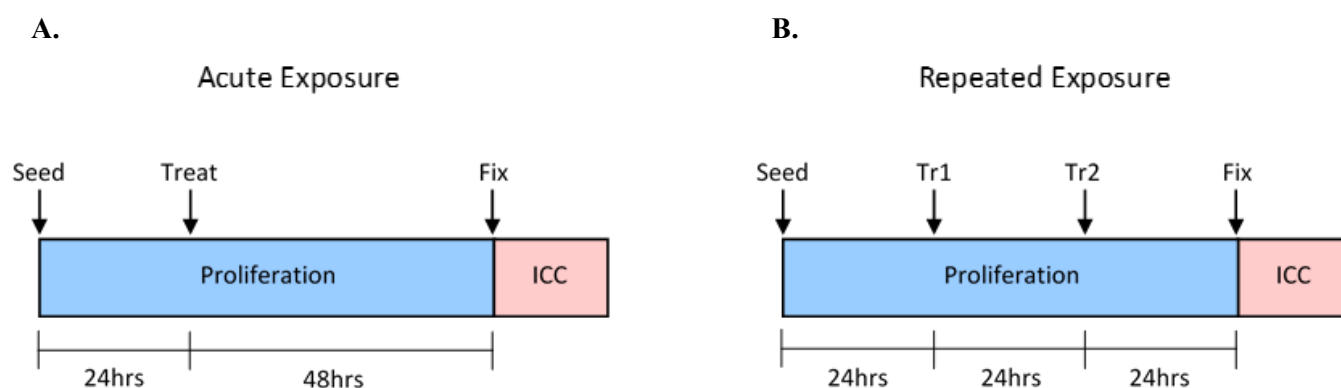
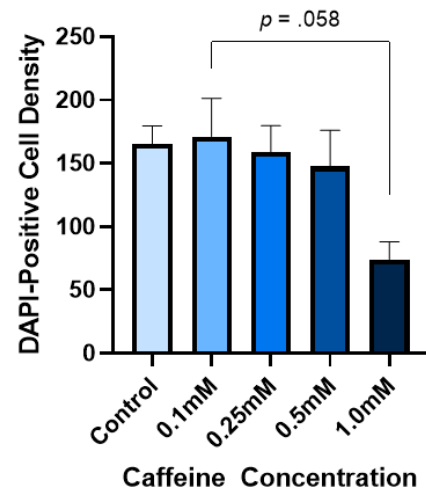


Figure 1. Schematic of the proliferation assays for the two caffeine exposure conditions. (A) Acute caffeine exposure. This plate received only one caffeine treatment, 24 hours after seeding. Treatment involved a full replacement of culture medium with 100µl caffeinated medium. **(B)** Repeated Caffeine Exposure. This plate received a treatment every 24hrs after seeding. Treatment 1 (Tr1) involved a full replacement of culture medium with 100µl caffeinated medium. Treatment 2 (Tr2) involved a ‘booster’ treatment, where 20µl of medium was removed and replaced with fresh caffeine medium. Booster treatments were made at 5x concentration. Both plates were fixed 72 hours after seeding. ICC; immunocytochemistry. Cell line: *HPC0A07/03*. Passage number: P21; Biological replicates: n=3; Technical replicates: n=3.

A.



B.

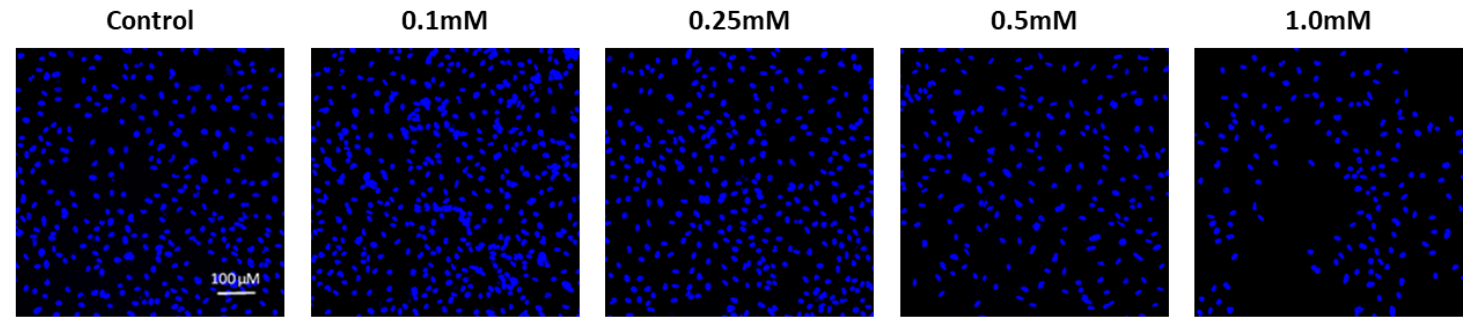
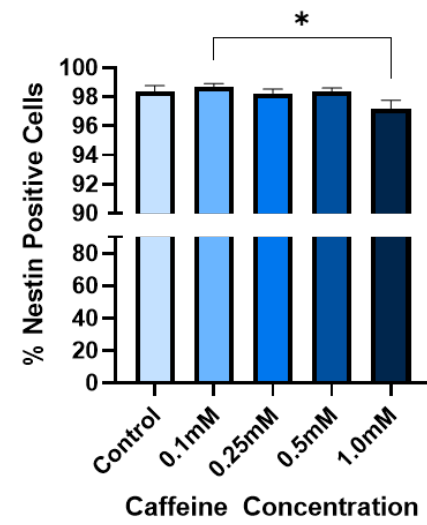
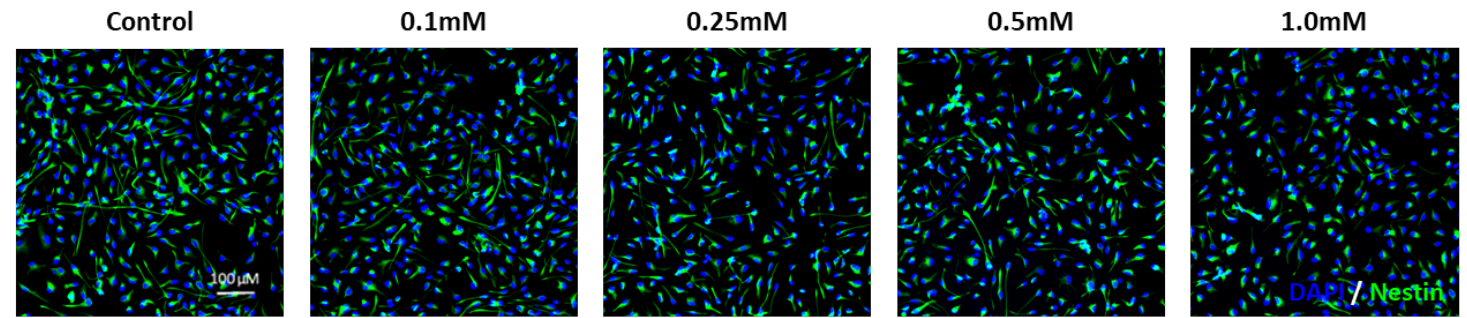


Figure 2. The effect of caffeine treatment on DAPI-positive cell density. (A). There was no significant interaction of caffeine concentration and exposure on DAPI-positive cell density (Two-way ANOVA: $F [4, 20] = .179, p = .947$), nor was there a significant main effect of exposure ($F [1, 20] = .317, p = .580$). However, caffeine had a significant main effect on DAPI-positive cell density (One-way ANOVA: $F [4, 25] = 3.041, p = .036$). Post hoc analyses revealed no significant differences in total cell number between any specific caffeine concentration and the control, nor between any caffeine concentrations, but a trend for significance emerged for the supraphysiological caffeine concentration (i.e., 1.0mM, $M = 74.13, SD = 34.27$) having reduced cell count compared with the lowest caffeine concentration (i.e., 0.1mM, $M = 171.12, SD = 74.21, p = .058$). (B). Representative immunostaining, demonstrating DAPI-positive cell density following exposure to different caffeine concentrations. Images taken at 10x objective; scale bar represents 100µM. Cell line: *HPC0A07/03*; Passage number: P21; Biological replicates: $n=3$; Technical replicates: $n=3$; Data represents the mean (\pm SEM); (adjusted p values; Bonferroni correction). Note: Graph not stratified by exposure given that no interaction or main effect of exposure was found.

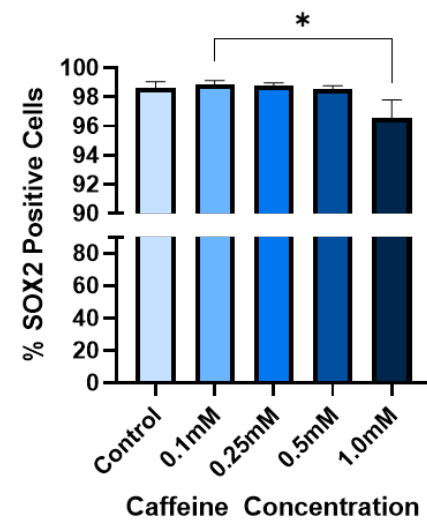
A.



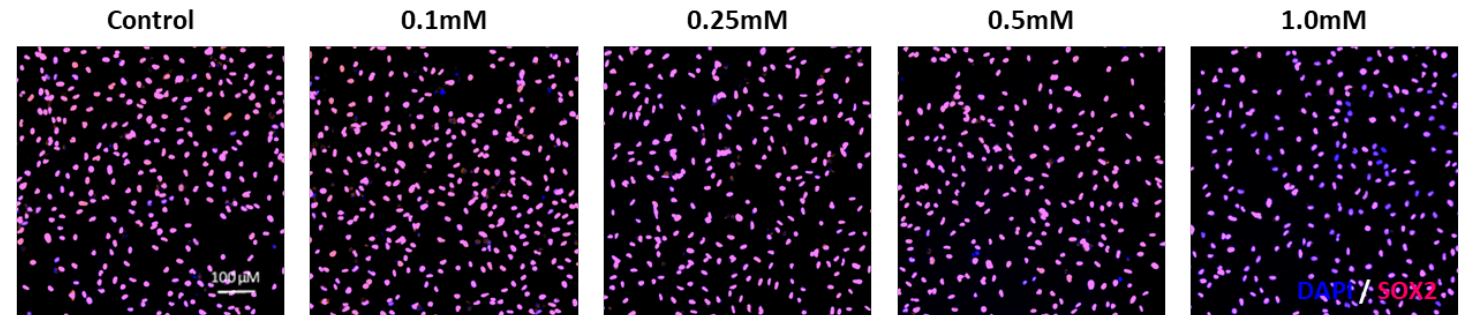
B.



C.



D.



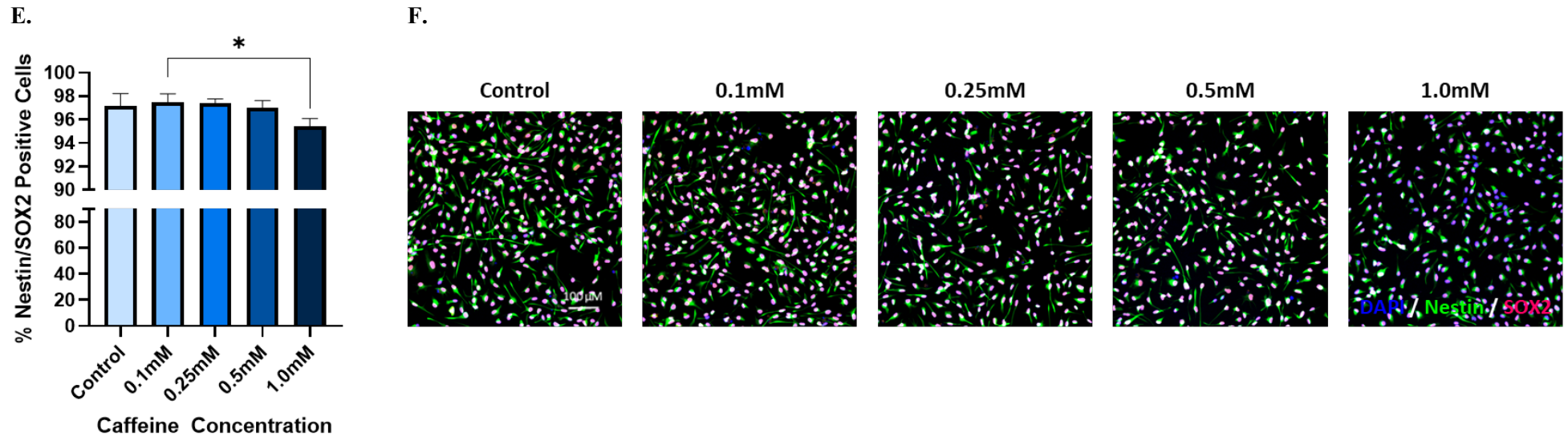


Figure 3. The effect of caffeine treatment on %Nestin-, %SOX2-, and Nestin/SOX2-positive cells. (A). There was no significant main effect of exposure on %Nestin-positive cells (Kruskal-Wallis test, $H = .02$, $df. = 1$, $p = .901$) but there was a significant main effect of caffeine (Kruskal-Wallis test, $H = 10.38$, $df. = 4$, $p = .034$). Dunn's post hoc analyses revealed that the supraphysiological caffeine concentration (i.e., 1.0mM; Mdn = 97.2, IQR = 1.99) had significantly reduced stem cell integrity compared with the lowest caffeine concentration (i.e., 0.1mM, Mdn = 98.66, IQR = .72, $p = .016$). (B). Representative immunostaining, demonstrating DAPI-positive cell density with %Nestin-positive cells following exposure to different caffeine concentrations. Images taken at 10x objective; scale bar represents 100µM. (C) There was no significant main effect of exposure on %SOX2-positive cells (Kruskal-Wallis test, $H = .01$, $df. = 1$, $p = .917$) but there was a significant main effect of caffeine (Kruskal-Wallis test, $H = 12.17$, $df. = 4$, $p = .016$). Dunn's post hoc analyses revealed that the supraphysiological caffeine concentration (i.e., 1.0mM; Mdn = 96.59, IQR = 2.8) was significantly reduced compared with the lowest caffeine concentration (i.e., 0.1mM, Mdn = 98.9, IQR = 0.81, $p = .013$). (D) Representative immunostaining, demonstrating DAPI-positive cell density with %SOX2-positive cells following exposure to different caffeine concentrations. Images taken at 10x objective; scale bar represents 100µM. (E). There was no significant main effect of exposure on %Nestin/SOX2-positive cells (Kruskal-Wallis test, $H = .03$, $df. = 1$, $p = .868$) but there was a significant main effect of caffeine (Kruskal-Wallis test, $H = 11.61$, $df. = 4$, $p = .021$). Dunn's post hoc analyses revealed the supraphysiological caffeine concentration (i.e., 1.0mM; Mdn = 95.46, IQR = 3.98) was significantly reduced compared with the lowest caffeine concentration (i.e., 0.1mM, Mdn = 97.48, IQR = 1.11, $p = .016$). (F). Representative immunostaining, demonstrating DAPI-positive cell density with %Nestin/SOX2-positive cells following exposure to different caffeine concentrations. Images taken at 10x objective; scale bar represents 100µM. %Nestin-, %SOX2-, and %Nestin/SOX2-positive cells are controlled for by DAPI. Graphs not stratified by exposure given that no main effect of exposure was found. Cell line: *HPC0A07/03*; Passage number: P21; Biological replicates: $n=3$; Technical replicates: $n=3$; Data represents the median (\pm IQR); * $p < .05$; (adjusted p values; Dunn's correction).

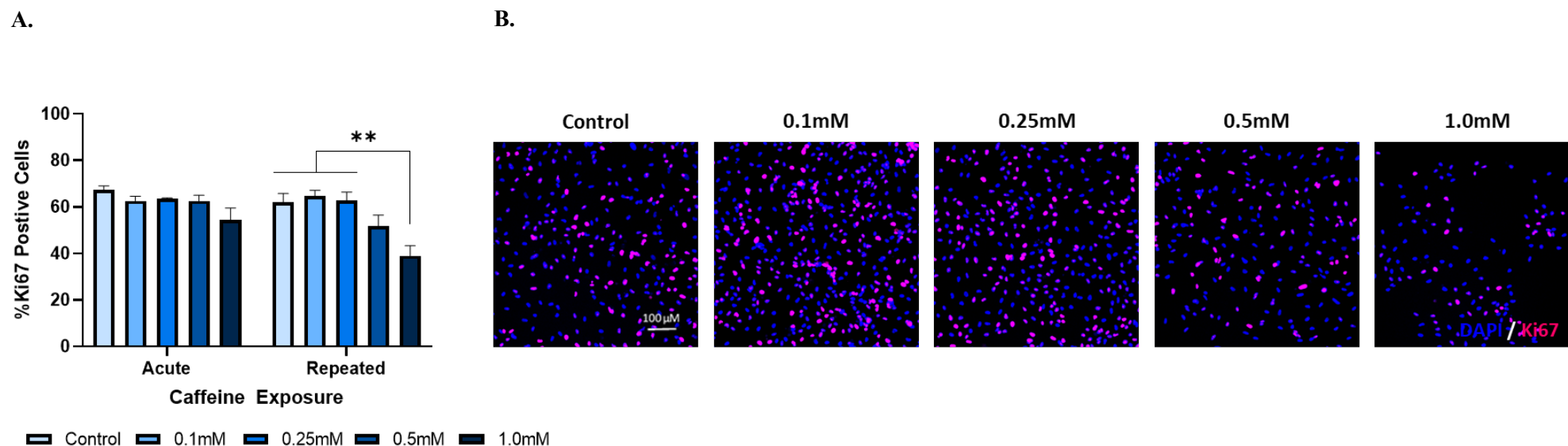


Figure 4. The effect of caffeine treatment on %Ki67-positive cells. (A) There was no significant interaction effect of caffeine concentration and exposure on %Ki67-positive cells (Two-way ANOVA: $F [4, 20] = 2.235, p = .102$). However, a significant main effect of both exposure (Two-Way ANOVA: $F [1, 20] = 8.292, p = .009$) and caffeine concentration (Two-way ANOVA: $F [4, 20] = 9.81, p < .001$) was found on %Ki67-positive cells. Specifically, Bonferroni post hoc analyses revealed that repeated treatment with the supraphysiological concentration (i.e., 1.0mM; $M = 39.05, SD = 7.5$) significantly reduced proliferation compared with the control ($M = 61.94, SD = 6.69, p = .001$), the lowest caffeine dose (i.e., 0.1mM; $M = 64.580, SD = 4.403, p < .001$), and the moderate caffeine dose (i.e., 0.25mM; $M = 62.68, SD = 6.35, p = .001$). (B). Representative immunostaining, demonstrating DAPI-positive cell density with %SOX2-positive cells following repeated exposure to different caffeine concentrations. Images taken at 10x objective; scale bar represents 100µM. %Ki67-positive cells are controlled for by DAPI. Cell line: *HPC0A07/03*; Passage number: P21; Biological replicates: $n=3$; Technical replicates: $n=3$; Data represents the mean (\pm SEM); ** $p < .01$ (adjusted p values; Bonferroni correction).

Supplementary Material

1 Supplementary Methods

1.1 Proliferation Assay

The hippocampal progenitor cell (HPC) proliferation assay was carried out as previously described (de Lucia et al., 2020; Smeeth et al., 2020). Briefly, HPCs were seeded into two 96-well plates (Nunc, Denmark) per experiment: one plate for acute (one-time) caffeine treatment, the other for repeated caffeine treatment. Plates were seeded at a density of 1.2×10^4 , at P21, with three technical replicates and three biological replicates. After seeding, cells were cultured in proliferation medium (reduced modified medium (RMM), with human epidermal growth factor (EGF), human basic fibroblast growth factor (bFGF), and 4-hydroxytamoxifen (4-OHT) for 24 hours, after which the media was replaced by caffeine supplemented proliferation media (i.e., full treatment) for 48 hours. The acute treatment condition was left undisturbed for 48 hours, while the repeated condition received another caffeine treatment 24 hours later, where 20 μ l of the caffeine supplemented proliferation media was removed and replaced with a concentrated caffeine dose (i.e., booster treatment). Control conditions were incubated in caffeine-free proliferation media in all instances. Seventy-two hours after seeding, plates were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Post-fixation, plates were washed and stored in PBS with sodium azide (NaN_3 ; 0.05% dilution) at 4°C in preparation for immunocytochemistry.

1.2 Caffeine Treatments

Prior to experimentation, caffeine concentrations were measured and dissolved in proliferation medium (RMM, with EGF, bFGF, and 4-OHT) separately for the two treatment types (i.e., full and booster). As full treatments involved completely replacing the proliferation media, these were made at 1x the intended concentration. For repeated exposure, booster treatments at 5x the intended concentration were used, and 20 μ l of the total 100 μ l media was replaced to prevent interfering with the cell-produced factors. All caffeine concentrations (i.e., full and booster) and control proliferation media were clarified, aliquoted and frozen at -4°C for future use. Since caffeine content has been shown to differ depending on brewing method (Bell et al., 1996), to minimize any changes in caffeine concentration, all caffeine and control aliquots were defrosted in the 37°C water bath for ten minutes and clarified before use. All aliquots were thawed only once prior to use.

1.3 Immunocytochemistry

To quantify proliferation and progenitor cell integrity, immunocytochemistry was used as previously described (de Lucia et al., 2020; Smeeth et al. 2020). Briefly, PFA-fixed cells were incubated for 1h at room temperature in blocking solution, consisting of PBS- NaN_3 with 5% normal donkey serum (D9963, Sigma) and 0.3% Triton-X (93443, Sigma). Cells were then incubated in 30 μ l of primary antibodies: Ki67 for proliferation, (Mouse anti-Ki67, 1:800, Cell Signalling Technology), Cleaved Caspase 3 for apoptosis (CC3; rabbit anti-CC3, 1:500, Cell Signalling Technology), and Nestin and SOX2 for hippocampal progenitor integrity (Mouse anti-Nestin, 1:1000, Merck Millipore; Mouse anti-Nestin, 1:1000, Abcam) - all diluted in blocking solution overnight at 4°C. Cells were then washed twice with PBS, and incubated in blocking solution for 30 minutes at room temperature,

before being incubated in 30µl of secondary antibodies (Alexa 488 donkey anti-mouse, 1:500, Life Technologies, A-21202; Alexa 555 donkey anti-rabbit, 1:500, Life Technologies, A-31572), diluted in blocking solution, for 2h at room temperature in the dark. Cells were then washed twice with PBS and incubated at room temperature for 5 minutes in 50µl of 300µM 4',6-diamidino-2-phenylindole solution diluted in PBS (DAPI; D9542-5mg, Sigma). Finally, cells were washed twice more before being stored in 200µl PBS with NaN₃ (0.05% dilution) at 4°C in the dark.

1.4 Image Analysis

In brief, images were taken with a 10x objective that autofocuses using the DAPI stain. Exposure time was manually defined to ensure a good signal to noise ratio. Individual cells were determined using the DAPI-positive nuclei, and smoothing, threshold, and segmentation parameters were modified as required to ensure accurate outlining of nuclei. Consequently, DAPI stains too small or large to be counted as nuclei were excluded.

Cellular markers of interest were then identified and quantified, with defined parameters that were dependent on each marker's location in relation to the nucleus. Nuclear proteins were indicated using a round target that overlaid the nuclear outline. Conversely, cytoplasmic proteins were indicated using a donut target, where the nucleus was denoted with the target's inner boundary. Average intensity thresholds (AIT) were manually defined, to distinguish specific fluorescent signals from unspecific binding and background noise. To set these, the experimental wells were compared to the negative control wells (i.e. without primary antibodies), and the threshold was set above the highest intensity signal of the negative control. Consequently, the positive cells (those with an AIT above the set threshold) could be differentiated from the negative cells (AIT below the set threshold). These parameters were kept constant throughout every experiment. Once the parameters had been set, plates were scanned. Fifteen fields per well were captured, in which the software calculates the percentage of all cells positive for each marker (identified by the appropriate cellular stain), as controlled for by DAPI-positive nuclei.

2 Supplementary Figures

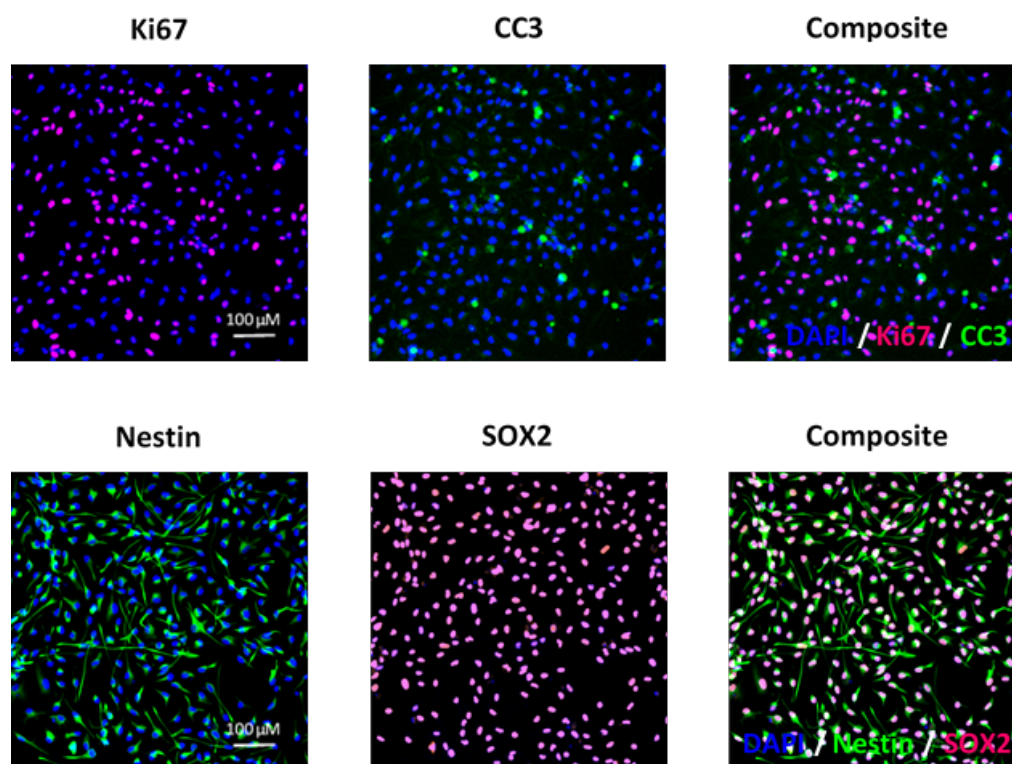
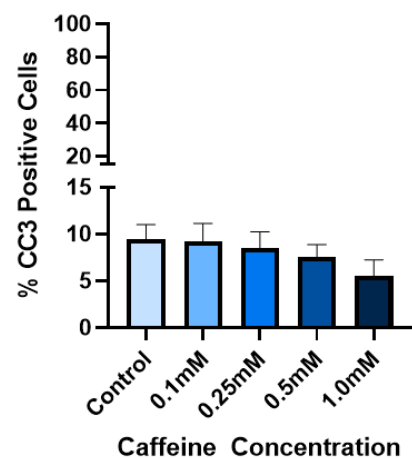
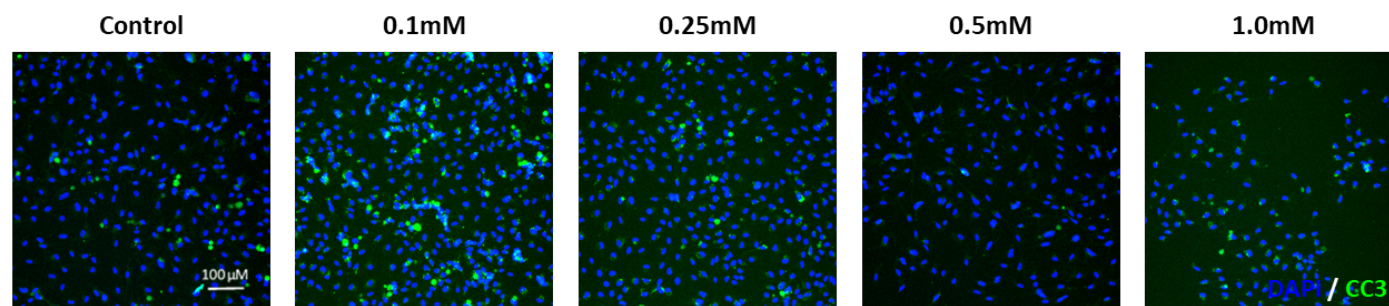


Figure S1. Representative images of immunostaining. Each box represents a field analysed by the Cell Insight software. Row 1 represents (from left to right) Ki67 in pink, CC3 in green, and Ki67/CC3. Row 2 represents (from left to right) Nestin in green, SOX2 in pink, and Nestin/SOX2. DAPI stainings are shown in dark blue in all images. Images taken with at 10x objective; scale bar, on the bottom left box, represents 100μM. Cell line: *HPC0A07/03*; Passage number: P21.

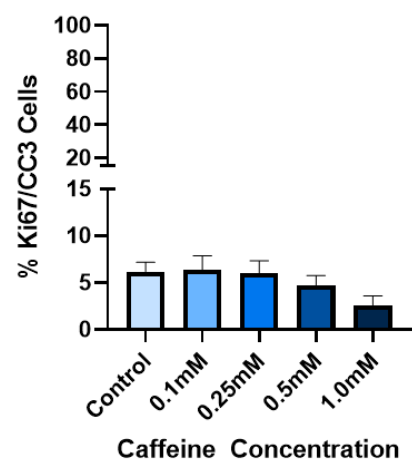
A.



B.



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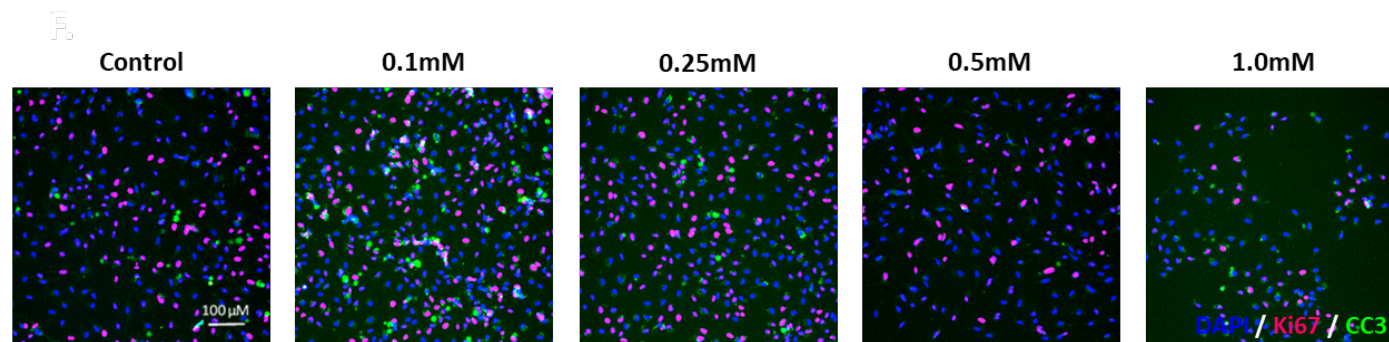


Figure S2. The effect of caffeine treatment %CC3- and %CC3/Ki67-positive cells. (A). There was no significant main effect of caffeine (One-way ANOVA: $F [4, 25] = 9.09, p = .474$), exposure (Two-way ANOVA: $F [1, 20] = .331, p = .571$), nor an interaction effect (Two-way ANOVA: $F [4, 20] = .677, p = .616$) on apoptosis, that is %CC3-positive cells. **(B).** Representative immunostaining, demonstrating DAPI-positive cell density with %CC3-positive cells, following exposure to different caffeine concentrations. Images taken at 10x objective; scale bar represents 100 μ M. **(C).** There was no significant main effect of caffeine (One-way ANOVA: $F [4, 25] = 1.767, p = .167$), exposure (Two-way ANOVA: $F [1, 20] = .097, p = .759$), nor an interaction effect (Two-way ANOVA: $F [4, 20] = .413, p = .797$) on %CC3/Ki67-positive cells. **(D).** Representative immunostaining, demonstrating DAPI-positive cell density with %CC3/Ki67-positive cells following exposure to different caffeine concentrations. Images taken at 10x objective; scale bar represents 100 μ M. %CC3- and %CC3/Ki67-positive cells are controlled for by DAPI. Graphs not stratified by exposure given that no main effect of exposure was found. Cell line: *HPC0A07/03*; Passage number: P21; Biological replicates: $n=3$; Technical replicates: $n=3$; Data represents the mean (\pm SEM) (adjusted p values; Bonferroni correction).

3 Supplementary References

Bell, L.N., Wetzel, C.R., Grand, A.N. (1996). Caffeine content in coffee as influenced by grinding and brewing techniques. *Food Res Int.* 29:785-789. [https://doi.org/10.1016/S0963-9969\(97\)00002-1](https://doi.org/10.1016/S0963-9969(97)00002-1)

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